REMARKS

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

Amendments to the Claims

Claims 20 and 21 are amended and new claims 34-37 are added. Support for amended claim 20 may be found, for example, in the specification in Example 6 and Figure 7, which discusses the relapse rate of patients treated with compositions as recited in the claims, e.g., comprising NY-ESO-1 and saponin based adjuvant. Claim 21 is amended to correct its antecedent basis. Support for new claims 34 and 35 may be found in the specification at paragraph [0007] which describes ISCOM and ISCOMATRIX adjuvants. Support for new claims 36 and 37 may be found, *inter alia*, in the specification at paragraphs [0090] and [0091], which describe routes of administration and amounts of NY-ESO-1 for administration. The amendments to the claims, therefore, do not introduce any new matter.

Claims 23, 24 and 27 are cancelled without prejudice or disclaimer.

Applicants reserve the right to pursue any canceled subject matter in one or more continuing applications with the same rights of priority as the instant application.

After amending the claims as set forth above, claims 20-22, 25, 26 and 34-37 will be pending in this application. Applicants respectfully request reconsideration of these claims.

Rejections under 35 U.S.C. § 112, 1st paragraph (enablement)

Claims 20-27 are rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the enablement requirement. Office Action at 2. Specifically, the Examiner maintains that the specification does not enable "fragment(s) or variants thereof of NY-ESO-1 protein, or homologues or polytopes of NY-ESO-1, with a saponin based adjuvant." *Id.* Applicants respectfully traverse this rejection.

Solely in an effort to expedite prosecution, and not in acquiescence to the propriety of the rejection, Applicants have amended claim 20, from which all other pending claims

depend, to recite that a composition comprising "full length NY-ESO-1 protein and a saponin" are administered to a subject. The Examiner has acknowledged that the specification is enabling for methods using "full length NY-ESO-1 protein and ISCOM adjuvant or ISCOMATRIX adjuvant... ." Office Action, at 2. Thus, Applicants respectfully request withdrawal of the enablement rejection.

Rejections under 35 U.S.C. § 112, 2nd paragraph

Claim 24 is rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite due to the recitation of the trademark names "ISCOM" and "ISCOMATRIX." Office Action at 5. Not acquiescing in the propriety of the rejection, and solely to advance prosecution, Applicants have cancelled claim 24 and added new claims 34 and 35 which describe the claimed saponin based adjuvants using generic terminology as set forth in the specification. Thus, Applicants consider the rejection moot.

Rejections under 35 U.S.C. § 102(b)

Claims 20-27 are rejected under 35 U.S.C. § 102(b) for allegedly being anticipated by Cebon *et al.*, *Proc. Amer. Soc. Clin. Oncol.* 21, abstract 86 (June 2002) ("Cebon"). Office Action, at 6. Applicants respectfully traverse.

The Examiner alleges that the claimed method "appears to be the same as the process of [Cebon] absent a showing of differences." Office Action at 6. Applicants disagree. Solely in an effort to advance prosecution, and not in acquiescence to the propriety of the rejection, Applicants have amended claim 20, from which all other pending claims depend, to recite "a method for reducing the risk of relapse in a subject at risk of relapse of a cancer, cells of which express NY-ESO-1." Thus, the presently claimed methods relate to a method for reducing the risk of relapse by administration of a composition comprising full length NY-ESO-1 protein and a saponin based adjuvant, to subjects at risk of relapse of a cancer, cells of which express NY-ESO-1.

Cebon does not teach or suggest methods of reducing the risk of relapse, and does not teach or suggest that a composition comprising NY-ESO-1 protein and a saponin based

adjuvant could or should be used to reduce the risk of relapse. Moreover, Cebon does not contemplate administration of a composition as claimed to the recited patient population, e.g., subjects at risk of relapse of a cancer, cells of which express NY-ESO-1.

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described in a single prior art reference," and "[t]he identical invention must be shown in as complete detail as is contained in the ... claim." (MPEP § 2131). Here, where Cebon fails to disclose a method as claimed, the §102 rejection is improper and should be withdrawn.

Furthermore, Applicants wish to bring to the Examiner's attention two sets of data which provide evidence of the unexpected results associated with the method of the present invention. Specifically, Example 6 of the specification shows that out of a total of ***nineteen*** patients who were treated with a composition as recited in the pending claims (NY-ESO-1 + ISCOM adjuvant, dose A, B & C), only ***two*** had relapsed after a median follow-up of 748 days. *See* paragraph [0048]. In contrast, ***six out of sixteen*** antigen-only treated patients relapsed, and ***five out of seven*** placebo-treated patients relapsed. *See* paragraph [0048].

The patients were followed for an additional year, and the time to relapse of patients receiving NY-ESO-1/ISCOM showed a significant difference ($p=0.02$) as compared to placebo patients. *See* Figure 7 and paragraph [0049]. After one year of additional follow-up, a total of five of nineteen subjects treated with a composition as claimed had relapsed, seven of sixteen antigen-only treated patients relapsed, and relapse in the placebo group remained at five out of seven. *See* paragraph [0050]. Such an effect on ***relapse*** could not have been expected from Cebon.

A further follow-up study provides additional evidence that the claimed method unexpectedly reduces the risk of relapse. The manuscript by Nicholaou *et al.*, "Improved survival and persistence of antigen-specific immunity in patients who had previously been vaccinated with NY-ESO-1 protein formulated in ISCOMATRIX," ("Nicholaou") (submitted herewith) reports superior relapse-free survival ***three years*** post-vaccination, further demonstrating the efficacy of the invention and the unexpectedly superior results it achieves.

Nicholaou reports that the vaccine is highly potent immunologically. Indeed, the study found that 10 of 14 patients who had been vaccinated in accordance with the invention (NY-ESO-1 + ISCOMATRIX) exhibited ***persistent*** immunity, while only 3 of 14 who had been vaccinated with antigen alone or placebo exhibited long term immunity. *See* Nicholaou at 3. As noted at page 14 of Nicholaou, there "appeared to be a highly significant reduction in relapse rates in those patients who received vaccine with ISCOMATRIX® adjuvant, the same group that had the best immune persistence."

As there is no teaching or suggestion in Cebon of a method of preventing relapse, and no indication that the claimed methods would achieve such dramatic, beneficial results in the context of preventing relapse, the results reported in the instant application and Nicholaou are evidence of unexpected results that further support patentability. Applicants therefore respectfully request withdrawal of the rejection based on Cebon.

Conclusion

Applicants believe that the present application is in condition for allowance, and favorable reconsideration is respectfully requested.

If there are any questions regarding this submission, or if any issues remain, the Examiner is invited to contact the undersigned by telephone in order to advance prosecution.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing or a credit card payment form being unsigned, providing incorrect information resulting in a rejected credit card transaction, or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date November 13, 2009

By

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Title: Improved survival and persistence of antigen-specific immunity in patients who have previously been vaccinated with NY-ESO-1 protein formulated in ISCOMATRIX®

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Running title: Persistence of NY-ESO-1 immunity in vaccinated patients

Keywords: NY-ESO-1, melanoma, cancer vaccines, immunotherapy, immune monitoring, immunoediting.

Statement of translational relevance

We have previously shown that recombinant NY-ESO-1 protein administered in ISCOMATRIX® adjuvant is highly immunogenic in patients with resected cancer. It was not previously known whether these immune responses were long lasting or whether they were of clinical significance. This study demonstrates that immune responses to NY-ESO-1 can last for several years and are enhanced by the use of ISCOMATRIX® adjuvant. We also provide long term follow up of our original vaccinated cohort, showing apparent improved relapse-free survival in patients receiving optimal vaccination. In patients who relapsed and who were able to be analysed, relapse was associated with failure of presentation of NY-ESO-1 antigen by tumor, suggesting that immunoediting of the tumor had occurred under the selective pressure of vaccine-induced immunity. These results have led to an ongoing randomised phase II study of NY-ESO-1/ISCOMATRIX® vaccine compared to ISCOMATRIX adjuvant alone in patients with high risk resected melanoma.

Abstract

Purpose: NY-ESO-1 protein formulated in ISCOMATRIX® adjuvant (NY-ESO-1/ISCOMATRIX®) results in a broad-based CD4+, CD8+ T cell and antibody immune response. We evaluated the relapse-free survival (RFS) and persistence of NY-ESO-1-specific immunity in previously vaccinated subjects.

Experimental Design: RFS was determined for 46 melanoma patients and immunity was measured in 28 with fully resected NY-ESO-1-expressing tumors (melanoma 25, breast 3) 303 -1155 (median=681) days after vaccination. Treatment cohorts previously received NY-ESO-1/ISCOMATRIX® at protein doses 10μg, 30μg or 100μg (n=14), NY-ESO-1 protein alone at 100μg (n=8) or placebo (n=6). Immune testing consisted of: antibody titers; circulating NY-ESO-1 specific T cells on day 14; skin test reactivity two days after intradermal protein (1μg) and peptides (10μg) restricted by HLA-A2: NY-ESO-$1_{157-167}$, NY-ESO-$1_{157-165}$ and HLA-DP4: NY-ESO-$1_{157-170}$.

Results: Clinical follow-up of the melanoma patients showed superior RFS in those vaccinated with NY-ESO-1/ISCOMATRIX®. Persisting immunity in ten of 14 recipients who had received the vaccine with ISCOMATRIX® adjuvant was observed, only three of 14 had long-term NY-ESO-1 immunity among those who received either NY-ESO-1 protein alone without adjuvant.Thus there was a clear association between persisting anti-NY-ESO-1 immunity and prior vaccination ($p<0.0067$). In participants who relapsed despite vaccination, down regulation of NY-ESO-1 or HLA Class I expression indicated immunoediting had occurred.

Conclusion: An apparent signal of increased RFS was observed in high-risk resected melanoma patients vaccinated with NY-ESO-1/ISCOMATRIX® adjuvant and persisting immunity to NY-ESO-1 was detected in the majority of vaccinated subjects tested. A prospective randomized trial is underway to confirm these results.

Introduction

NY-ESO-1 is a cancer testis (CT) antigen that has attracted particular attention as a candidate antigen for cancer vaccine strategies (*1-3*) or more recently for adoptive transfer of T lymphocytes (*4*). It was initially identified by serological analysis of recombinant cDNA expression libraries (SEREX), using tumor mRNA and autologous serum from a participant with squamous cell carcinoma of the esophagus. The full-length cDNA encodes a protein of 180 amino acids (*1*). NY-ESO-1 is an attractive candidate for cancer vaccine development because of its expression in a wide variety of human cancers including melanoma, and because of the frequency of spontaneous anti-NY-ESO-1 immunity in cancer-bearing patients. Furthermore, normal expression is restricted so that it is only found in the germ line cells in testis, oogonia and in the placenta, all of which are immune-privileged sites(2, 3). These characteristics suggested that NY-ESO-1-based vaccines are likely to be immunogenic and that the resulting immune responses would unlikely be directed against normal tissues.

Although NY-ESO-1 was initially identified on the basis of serological recognition, NY-ESO-1 peptides are also recognized by autologous cytotoxic T lymphocytes (CTL).The first HLA class I-restricted peptides described were an overlapping group presented by HLA-A2: SLLMWITQCFL (position 157-167; referred to as NY-ESO-1a), SLLMWITQC (position 157-165; referred to as NY-ESO-1b), and QLSLLMWIT (position 155-163; referred to as NY-ESO-1c)(*5*). For many years, these HLA-A2-restricted epitopes were thought to be immunodominant. However many additional peptides restricted by HLA-B and HLA-C molecules were subsequently identified as epitopes against which spontaneous immunity develops, or in response to vaccination with full length antigen (*6-11*). Furthermore, numerous helper epitopes presented by MHC class II molecules have also been found (*12-14*).

HLA class I- and II-restricted epitopes have been identified along the full length of the NY-ESO-1 protein with a "hot spot" around the central region of the NY-ESO-1 protein from amino acids 80 – 110 (*10, 13*). T-cell responses specific to epitopes in this region in many patients with HLA-A2 expression appear more common than those restricted to HLA-A2/NY-ESO-$1_{157-165}$ epitope (*11*). The region comprising amino acids 157-170 was the first HLA class II-restricted epitope described. This peptide, restricted by HLA-DP4, was reported to correlate with antibody production and therefore proposed to be important for providing CD4+ T cell-mediated immune responses (*15*) although a more recent study found no correlation between HLA-DP4 status and antibody response(16). This longer peptide also incorporates the HLA class I-restricted NY-ESO-$1_{157-167}$ and NY-ESO-$1_{157-165}$ peptides. Subsequently many additional epitopes recognized by CD4 T cells in the context of HLA-DR alleles have been identified.(12, 13, 15, 17-21). A current list of HLA class I and II epitopes is found at http://www.cancerimmunity.org/peptidedatabase/tumorspecific.htm

We previously performed a placebo controlled clinical trial LUD99-008 (initial trial) with the full length NY-ESO-1 protein with the aim of evaluating the safety and immunogenicity of NY-ESO-1 vaccination in forty-six evaluable

participants with fully resected NY-ESO-1 positive tumors (*10*). NY-ESO-1 was formulated with ISCOMATRIX® adjuvant (22) (CSL Limited, Australia) and the protein dose was escalated in three cohorts 10 µg/dose (cohort A), 30 µg/dose (cohort B) and 100µg/dose (cohort C). Each participant received three doses of vaccine intramuscularly at monthly intervals. Additional cohorts received protein alone without ISCOMATRIX® adjuvant (cohort D) or placebo (Normal Saline for Injection) (P). all cohorts received 1 µg of intradermal NY-ESO-1 protein for delayed-type hypersensitivity (DTH) skin testing, prior to vaccination and at day 84 after three vaccinations. The placebo group was included to control for this exposure to NY-ESO-1 recombinant protein.

The vaccine was safe and immunologically potent (*10, 13*). The majority of vaccinated participants achieved high-titer antibody responses, strong delayed-type hypersensitivity reactions and circulating CD8+ and CD4+ T cells specific for a broad range of NY-ESO-1 epitopes, including known and previously unknown epitopes. Weaker immune responses were seen in those who received either placebo or protein alone without ISCOMATRIX® adjuvant.

Participants in the initial trial had fully resected cancers but were at high risk of disease recurrence. There was concern that immune responses generated during that trial might not be sustained long-term without ongoing or repeated exposure to the antigen and no information was available regarding the persistence of NY-ESO-1-specific immunity following vaccination. The principal objective of the follow-up trial that is the subject of this report (LUD2001-017) was to characterize antigen specific responses in vaccine recipients.

Immune responses in the initial clinical trial were generated against full-length protein and immune monitoring was based on T-cell-mediated immune responses measured in blood. We were interested in knowing whether skin testing with intradermal protein or peptides could be used as a simple alternative means of measuring cellular immune responses to individual epitopes in participants who had previously been vaccinated with the full-length protein. We sought to validate these skin reactions by correlating them with the NY-ESO-1-specific T cell responses detected in peripheral blood.

Additionally, striking cutaneous immune reactions to NY-ESO-1 protein were observed in some participants, with up to 35mm of induration. However the NY-ESO-1 protein was produced in a bacterial expression system so immune responses against small amounts of contaminating bacterial protein may have contributed to some skin reactions to full-length protein. Use of fully synthetic peptides would allow further refinement of the specificity of skin testing.

The follow-up trial reported here was therefore undertaken to determine whether NY-ESO-1-specific immunity persisted after vaccination; and if so, whether it was possible to characterize persistent immunity further. Skin testing with selected peptides was assessed as a simple method for evaluating responses against NY-ESO-1-specific CD4+ or CD8+ epitopes following previous protein vaccination.

During the course of this trial, we made an additional and unexpected observation: participants receiving the most effective form of the vaccine appeared to be relapsing at a lower frequency than those who received placebo or NY-ESO-1 protein alone without ISCOMATRIX® adjuvant. An unplanned analysis of relapse free survival (RFS) was therefore performed to assess this. The results of this survival analysis are reported here.

Materials and Methods

Participant Population

Participants eligible for the follow-up trial (LUD2001-017) had all previously completed participation in the initial trial (LUD99-008). Inclusion criteria included an expected survival of at least one month; adequate major organ function; and written informed consent. Patients were not required to be disease free at entry into the follow-up trial and those who had relapsed between the two trials were eligible to receive vaccination if this was deemed to be an acceptable clinical option by the treating physician. Exclusion criteria included: concomitant immunosuppressive therapy, immunodeficiency or HIV; other serious illnesses; metastatic disease to the central nervous system, unless treated and stable; recent chemotherapy, radiation therapy or immunotherapy; participation in another clinical trial involving another investigational agent within 4 weeks of enrolment; pregnancy or lactation and inability to give informed consent. The protocol was approved by the Ludwig Institute for Cancer Research Protocol Review Committee and the Austin Health Human Research Ethics Committee, and all participants provided written informed consent. The study was independently monitored by Kendle Australia. The study was performed prior to the requirement for trials to be registered on an ICMJE-compliant registry.

Trial Design

Trial LUD2001-017 was an open-label single-arm study. All participants were assigned sequentially to the treatment regimen. The HLA class I and II peptides and NY-ESO-1 protein were administered ID on a single occasion at separate sites 10cm apart. Of the first eight participants, only one reaction to the intradermal peptides was seen. Consequently, the protocol was amended so that the intradermal dose of NY-ESO-1 protein was administered four weeks prior to the peptides thereby acting as a potential boost prior to skin and blood immune testing. This ensured that all subsequent participants were assessed at the same time point after most recent exposure to NY-ESO-1 recombinant protein.

Blood samples were obtained for the assessment of NY-ESO-1-specific antibodies by ELISA, NY-ESO-1-specific T cells and for laboratory safety assessments. Blood draws for immunologic assays were performed at baseline and two weeks following peptide injection. Skin reactions (induration and erythema) were measured 48 hours after the intradermal injection, and some reactive injection sites were biopsied for histopathology. Toxicity was documented according to National Cancer Institute Common Toxicity Criteria version 2.0, April 30, 1999.

Protein and peptides.
Peptides for skin testing: Three class I peptides restricted by HLA-A2 were NY-ESO-$1_{157-167}$ (NY-ESO-1a) (SLLMWITQCFL), NY-ESO-$1_{157-165}$ (NY-ESO-1b) (SLLMWITQC), and $gp100_{280-288}$ (gp100) (YLEPGPVTA). One class II peptide restricted by HLA-DP4 was: NY-ESO-$1_{157-170}$ (DP4) (SLLMWITQCFLPVF). All peptides were manufactured by Multiple Peptide Systems (MPS, San Diego, CA) to GMP specifications. Peptides were characterized by quantitative amino acid analysis and mass spectrometry by M-Scan Limited, England (DP4) or Auspep Pty. Ltd., Melbourne, Australia (gp100, DP4 and NY-ESO-1a/1b) . Biological safety testing for all peptides was conducted by BioReliance Inc., Rockville, Maryland, USA and passed all tests of sterility/endotoxin detection and bacteriostatic/fungistatic activity. 10μg of each was delivered by intradermal injection.

NY-ESO-1 protein was produced by fermentation carried out at CSL Limited, Melbourne, Australia and purified and formulated in conjunction with the Ludwig Institute For Cancer Research, Melbourne branch, Australia (23). The final NY-ESO-1 protein concentration was 0.3mg/mL in 4M urea and 50mM glycine; 15mM Sodium Chloride, 100mM Phosphate buffered saline, pH 6.5. For skin testing, a 1μg dose was administered intradermally.

Immunologic assays
The immunological responses were determined by using standardized assays:

Serology: NY-ESO-1-specific antibodies were measured by ELISA using a standardized and validated assay previously described (*10*). Blood was drawn at baseline and two weeks after NY-ESO-1 protein administration. Sera from the previous study initial were re-assayed concurrently to enable direct comparisons of antibody titer.

Skin tests: Peptides (10ug/dose) were injected ID on a single occasion either concurrently with the NY-ESO-1 protein dose (n=8) or 4 weeks after the protein dose (n=20). To establish a baseline for NY-ESO-1 peptide cutaneous reactivity, a series of controls were obtained from 15 NY-ESO-1 vaccine-naïve participants who had participated in two other Ludwig Institute-sponsored trials, LUD2002-003 n=9 (unpublished) and placebo participants n=6. For the peptides, induration following intradermal injection of DP4, NY-ESO-1b, NY-ESO-1a peptides was 0mm (mean + 2SD). Protein responses in the initial trial were based on an examination of blinded data with pre-existing reactivity defined as a baseline induration of >5mm. A positive response to vaccination was recorded if the second measurement was >5mm and at least double the baseline reading. In placebo 'vaccinated' participants, injection of 1μg or protein alone at baseline produced induration of 6mm (mean + 2SD). A positive response to vaccination was therefore defined as follows: with peptide any induration >0mm was recorded as a positive response. For protein induration >5mm was deemed to be positive and if it remained at least twice the baseline value in the initial trial was regarded as a persistent

response. To reduce variability, the administration of skin testing reagents and interpretation of responses was limited to a small number of personnel.

T cell assays: Flow-cytometric assays were based on intracellular cytokine staining (ICS) as previously described and epitopes mapped using overlapping peptides (*10*). For the ICS assay T cells were expanded over a 10-14 day period using the appropriate NY-ESO-1 18-mer peptides. Peripheral blood mononuclear cells ($5x10^6$) were pulsed with 10μM 18-mer peptides in pools of 3x 18mers for one hour at 37°C in 200μL RPMI +10% FCS (RF10) using a 24 well plate. An hour later, RF10 + 25IU IL-2 was added to 2.4mL. Cultures were fed and/or split every 2-3 days and a response first screened on day 10. An internal control (EBV-specific T cells) for T cell expansion efficiency was also set up in parallel. Positive controls during the readout phase of the ICS assays were newly thawed T cells with known specificity. T cells that were not stimulated with peptide in the assay served as negative controls.

Further examination of CD8+ and CD4+ T cell responses to undefined T cell epitopes were performed using NY-ESO-1 13-mers overlapping by 11 amino acids using ICS. 18-mer peptides were individually synthesized, and 13-mers were synthesized as cleaved pin-peptides (Chiron Mimotopes, Victoria, Australia). Data are expressed as percent positive staining of CD8+ or CD4+ T cells.

Relapse Free Survival: Survival data were collected with specific ethical approval and data were monitored for accuracy by Kendle Australia. The analysis was restricted to the vaccine recipients with a diagnosis of melanoma who were previously vaccinated with NY-ESO-1 (cohorts A, B and C of the original trial) and concurrent placebo recipients(10). RFS was calculated from the time of entry into the initial study LUD99-008. Patients without progression were censored at the date they were last known to be disease free.

Immunohistochemistry

Immunohistochemistry for NY-ESO-1, S100 and HLA I expression was performed as previously described (24).

Statistical considerations

The study population was drawn from participants in the previous initial trial who were available and willing to participate and who were eligible. The relationship between prior vaccination and persistent immunity was determined using Fishers exact test. A Kaplan Meier curve was plotted and the impact of vaccination was determined using Cox proportional hazard model.

Results

Participants

Twenty-eight of 46 patients from the original initial study entered the follow-up trial. Of the 18 not entered into the study, eight were dead, two were on corticosteroids, two were geographically inaccessible, and six declined. The demographics of the participants in the follow-up trial are shown in Table 1. Fourteen of these had received vaccination with protein formulated with ISCOMATRIX® adjuvant (cohorts A: vaccine dose 10ug, B: vaccine dose 30ug and C: vaccine dose 100ug) and 14 had been treated with either protein alone or placebo (cohorts D:NY-ESO-1 protein 100ug without ISCOMATRIX® adjuvant and P: Placebo, respectively). Participants in cohorts D and P had no NY-ESO-1 immunity or poor quality NY-ESO-1 responses at the completion of the earlier trial (10). Eleven of the 28 participants relapsed in the interval between the two studies and two patients had evident disease at study entry that did not require standard treatment at the time. The remainder had no evidence of disease.

Safety

There were no serious adverse events reported for this study. Minor (grade 1 and 2) adverse events including fever and injection site pain were reported in relation to administration of NY-ESO-1 protein and peptides. No grade 3 or 4 events were observed, which is consistent with adverse events reported previously (10).

Immune reactions to protein or peptide injections.

Antibody (Ab) to NY-ESO-1. Figure 1A shows anti-NY-ESO-1 antibody results from the two trials 252 – 1155 days apart. Only 3/15 patients remained seropositive at the time of entry into the follow-up trial. One of these (patient 1 from cohort A) had spontaneous NY-ESO-1 immunity at the time of entry into the initial clinical trial associated with clinical evidence of persistent indolent NY-ESO-1-expressing melanoma upon entry into the follow-up trial. Following the administration of NY-ESO-1 protein and peptides in the follow-up trial four participants had an increase in antibody titer. Three of these had previously been vaccinated in the cohort who received the highest dose of vaccine with ISCOMATRIX® adjuvant (cohort C) and the fourth receiving protein only (cohort D). Two participants had no evidence of antibody at study entry (cohort C) whereas two had persisting antibody titers (cohort A and C).

Skin reactions to NY-ESO-1 protein. Figure 1B shows the reactions to the 1μg i.d. NY-ESO-1 protein at three time points; (i) prior to vaccination on initial, (ii) at the completion of dosing on that protocol and (iii) 252-1155 days later upon re-challenge. With the exception of two participants skin reactions declined between studies. Diameters fell from 8.1+/-1.7 to 4.3+/-1.3 mm. Twelve participants had persistently negative skin test reactions (six in cohort D, four placebos, one from A and one from B). All four with a persistent cutaneous reaction were from cohort C of the earlier trial at which time DTH responses were marked (Figure 1B). One of these had relapsed in the intervening period. Additionally one patient who had previously only received placebo developed a de novo reaction in the absence of apparent clinical relapse. Two participants who had persistent tumor had no cutaneous reactions to protein.

There was also no apparent relationship between a persistent or increase in antibody titer and skin reaction to NY-ESO-1.

Skin reactions to NY-ESO-1 peptides

Only one peptide-specific cutaneous reaction was observed among the first eight participants accrued to the follow-up trial. Since we had anticipated DTH memory responses in these, we considered that it may be necessary to 'boost' cellular immunity in order to elicit these reactions. Consequently the protocol was amended and the subsequent 20 participants received the 1μg protein dose four weeks before cutaneous testing with the peptides. Table 2 shows the protein and peptide specific reactions among these. In general peptide-specific response were modest and correlated poorly with responses to protein, suggesting that cellular immune responses to these particular peptides were unlikely to be making a significant contribution to the inflammation that was induced by the whole protein. Failure to demonstrate a peptide response may have been due to HLA mismatch; for example, those who were HLA-A2 negative would not be expected to respond to peptides restricted by HLA-A2, however responses were inconsistent with our expectations; and three HLA-A2 negative patients responded to the NY-ESO-1b peptides (patients 23, 25 and 28). This may be due to the 1μg dose being too small to reactivate the necessary cellular and therefore humoral responses. There was a reduced reportoire of Ag phenotypes that may have also had an influence. In addition, some HLA-A2 positive patients responded to NY-ESO-1b but not NY-ESO-1a (patients 3 and 9). Since the NY-ESO-1b sequence sits within the sequence of the longer NY-ESO-1a peptide, there was an expectation that NY-ESO-1b responders would also be able to mount a response to NY-ESO-1a; for unknown reasons, however, this was not the case. Taken together with the assays for circulating T cell responses (Table 2 and Figure 2), we concluded that skin testing with these peptides did not give a reliable indication of anti-NY-ESO-1 T cell immunity.

Cellular immune responses to NY-ESO-1. T cell responses against peptides were evaluated on a subset of 16 participants. These were selected based on the availability of stored blood samples remaining from the initial clinical trial because we wanted to concurrently compare responses between studies. Table 3 shows the summary data for DTH, antibody and T cell responses in all patients. Figure 3 shows epitope-specific responses from both trials in those patients who were found to have persisting T cell immunity by ICS, together with DTH responses to protein in mm. Persisting immunity to both CD4 and CD8 epitopes were documented in most (12/16) participants tested 303-932 days after completing the initial study. The majority of these were treated in the initial trial in cohort C; NY-ESO-1 protein 100μg combined with ISCOMATRIX® adjuvant.

Relationship of long-tern immunity with vaccine cohort

Table 3 shows that there was a clear relationship between persistent immunity and treatment cohort from the initial trial. Of the thirteen participants who had persisting immunity, ten had received vaccine with ISCOMATRIX® adjuvant and three had not (these received either protein alone or placebo). Of those three, one had relapse melanoma and it is not possible to know

whether immunity in this case reflects the effect of vaccination or the induction of a spontaneous immune response to NY-ESO-1 antigen arising from the relapsed melanoma. For those patients without immunity, the majority (12/15) did not receive vaccine with ISCOMATRIX® adjuvant. Consequently persisting immunity was strongly related to vaccination with ISCOMATRIX® adjuvant in either cohort A, B or C (p= 0.007, Fishers exact test).

Clinical outcomes

During the course of this trial, we gained a strong clinical impression that clinical relapse in this group of patients may have been related to treatment cohort. To assess the effect of treatment cohort on relapse free survival all 46 melanoma patients were evaluated. With a median follow-up of 1430 days, an apparent effect of vaccination was observed for the NY-ESO-1 ISCOMATRIX® vaccine recipients (cohorts A, B or C) when compared with those who were concurrently randomized to receive placebo with cohorts C and D(9). The hazard ratio for RFS was 0.109 (95% CI: 0.003 – 0.114, Figure 3A). Allocation to the treatment groups was not sequential and not randomized, except for randomization within cohorts C and D where eight patients received placebo. It is therefore possible that mismatches of prognostic factors between the groups accounted for the differences in outcomes. However, a retrospective assessment of prognostic factors (age, sex, primary lesion thickness, time since diagnosis, stage at study entry, number of recurrences prior to entry, time since last resection prior to study entry) did not reveal any apparent differences between these groups. In the vaccine group 12 were male, median tumor thickness at diagnosis was 1.8 mm, ulceration was present in 5/19 and proportion estimated to have a greater than 50% risk of relapse was 13/19 (68%). In the placebo group two were male, median tumor thickness at diagnosis was 2.2 mm, ulceration was seen in 2/7 and the proportion estimated to have a greater than 50% risk of relapse was 3/7 (43%).

Immunoediting

It was possible to biopsy relapsed melanoma in several relapsing patients who were treated with vaccine plus ISCOMATRIX® adjuvant. In each case loss of either HLA class I or NY-ESO-1 protein expression was demonstrated by immunohistochemistry (Figure 3B). This strongly suggested that the vaccine was effective at inducing selective pressure on the tumor phenotype.

Discussion

There is growing recognition of the dynamic response between immunity and cancer, described recently in three stages; "Elimination", "Equilibrium", and "Escape"(25). Unless total elimination is achieved as a result of initial therapy, effective anticancer immunotherapy will require persistence of an immune response in order to sustain the equilibrium of remission status. It is therefore critical to understand and document vaccine efficacy, not only in terms of the initial induction of an immune response, but also in terms of the durability or longevity of that responses.

We therefore sought to evaluate a cohort of patients who had previously been vaccinated with a cancer vaccine which proved to be highly effective at inducing cellular and humoral immune responses against NY-ESO-1. The patients examined in this study had been vaccinated more than three years previously on a protocol that included several protein dose levels in conjunction with ISCOMATRIX® adjuvant, as well as protein alone without ISCOMATRIX® adjuvant, or placebo. We show here that NY-ESO-1-specific immunity persisted long-term, especially in those patients who received vaccine formulated with the ISCOMATRIX® adjuvant. Immunity could be boosted following re-challenge with NY-ESO-1 protein, and rechallenge with antigen was safe and well tolerated. For those patients who received the optimal vaccine preparation there has been a strong signal of improved relapse free survival, an observation which warrants validation. Finally, in those patients whose disease 'escaped' immune control, loss of NY-ESO-1 antigen or HLA class I point to immune evasion through down-regulation of the target antigen complex.

Very little is known about the long term persistence of vaccine-induced immunity following immunization with full length protein-based cancer antigens. We observed T-cell responses in 11/15 patients tested 303-932 days from the last exogenous NY-ESO-1 exposure. These responses were characterized by readily detectable CD4+ and CD8+ lymphocyte responses elicited following *in vitro* restimulation with peptides. In most cases, the specificity of the responses elicited during initial vaccination were detected although in some instances, responses to new specificities appeared (Figure 3). In contrast, cutaneous responses and antibody titers were either attenuated or lost in the period between the initial and follow-up studies (median time 680 days [range 252-1155 days]; figure1A & B).

Other investigators have reported long-term follow up after peptide vaccination. Chiong et al described persistence of HLA class-1/peptide tetramer-binding T cells specific to a gp100 epitope 36 months after vaccination in five melanoma patients with minimal residual disease who remained free of disease for more than 4.5 years (26). It is likely that these responses were vaccine-induced, however baseline values were not reported and there is a possibility that some may have been spontaneous. Another report in patients with epithelial ovarian cancer vaccinated with NY-ESO-1 peptides characterized the longevity of CD8+ and CD4+ responses (27). Patients were selected on the basis of being disease free at least six months

post vaccination and all had demonstrable vaccine-induced responses at early time points. CD4+ T cells were detected in five of five patients six and 12 months following peptide (NY-ESO-$1_{157-170}$) administration. However only two patients had detectable CD8+ T cells at six and 12 months (K Odunsi, personal communication).

While previous trials have used intradermal testing to assess cellular immunity to tumor antigens (27-32), in most cases peptide vaccines were used for both treatment and monitoring responses to the peptide immunogen itself. Subtleties in reactivity to synthetic peptides or their contaminants may have influenced these results. In contrast, our study used recombinant full-length protein for the immunogen and synthetic peptides for assessments of class I and II peptide specific reactions.

The protocol was initially designed to assess skin test reactions to injected antigen without any attempt to 'boost' reactions before this testing. It was modified after the first eight patients because only one responded and subsequently the 1μg protein dose was administered four weeks before the peptides in the remaining patients. Following this amendment, skin reactions to the HLA-A2-restricted peptide were seen in 4/11 HLA-A2+ve patients, and to the DP4 peptide in 1/22 DP4+ve patients. The attempt to boost cellular immune reactions using protein was therefore unsuccessful. In retrospect this may be because the 1μg dose was too small to reactivate the necessary responses.

To confirm the specificity of the intradermal protein skin reaction, lymphocytes were obtained from a skin biopsy of protein intradermal injection site in one participant. Whilst on the initial study the cutaneous reaction to protein measured 60mm of induration on 569 days later on the current study, measured 30mm. The specificities of T cells isolated from this biopsy have been reported previously and confirmed the presence of CD8+ and CD4+ T cells specific for NY-ESO-1 peptides (*10*). These included the NY-ESO-1a/b peptides, which induced 3-4 mm of induration in the current trial.

Figure 2 provides a comparison between the cellular immune responses elicited in each of the trials. These assays, which rely on *in vitro* re-stimulation, were not quantitative. Nonetheless responses were generally weak in the 017 study (not shown), consistent with a tapering-off of the responses despite the persistence of immune memory. In general the epitopes that dominated the responses the first time around were the same as those seen years later. It remains possible that tumor antigen present in micro-metastatic disease could stimulate immunity even though disease was undetectable. The capacity of the immune response to sculpt the tumor phenotype is well recognised and has been termed 'immunoediting'(33). Perhaps tumor has the capacity to sculpt the immune response in a reciprocal manner.

Whilst the focus of these trials was on immunity and its persistence, an emerging clinical impression, stimulated us to review clinical outcomes retrospectively. In this analysis two stark observations were made. Firstly

there appeared to be a highly significant reduction in relapse rates in those patients who received vaccine with ISCOMATRIX® adjuvant, the same group that had the best immune persistence. Secondly, in those patients who relapsed despite vaccination, tumor immunoediting was apparent, with loss of either NY-ESO-1 or HLA class I (Figures 3A & B). This implies that vaccine-induce immunity not only persisted, but that it was capable of applying substantive selective pressure resulting in altered tumor phenotype. Superior clinical and immune outcomes in the group who received NY-ESO-1 formulated with ISCOMATRIX® adjuvant points to the efficacy of this adjuvant for stimulating immunity, and for generating immunity against clinically relevant NY-ESO-1 epitopes. In recent studies we have shown that ISCOMATRIX® assists cross presentation of NY-ESO-1 to generate T cell responses against epitopes that are targets for immune recognition on cancer cells as well as DC (Nicholaou et al. submitted). This may prove important, since differing or alternative antigen processing pathways can be accessed in professional Antigen Presenting Cells (APC) and tumors(34). For effective immunity to occur, antigen processing by APC needs to generate the same epitopes that serve on tumor target cells. Clinical efficacy suggests that this is occurring in the case of this vaccine.

To confirm the observations reported here, a randomised placebo controlled trial of NY-ESO-1 ISCOMATRIX® adjuvant in patients with stage III and resected stage IV melanoma has been initiated (LUD2003-009, clinicaltrials.gov identifier NCT00199901). Results from that trial are awaited with interest.

Acknowledgements

The Investigators are members of the Cancer Vaccine Collaborative. We acknowledge the invaluable assistance of the staff of the Ludwig Institute for Cancer Research, Melbourne, Australia, Tina Cavicchiolo and our research nurses and clinical fellows. Support for clinical trial LUD 01-017 was generously provided by the Cancer Vaccine Collaborative and Cancer Research Institute, NY. IDD was supported by an Australian National Health and Medical Research (NHMRC) Council Career Development Award and currently by a Victorian Cancer Agency Clinician Researcher Fellowship and an honorary NHMRC Practitioner Fellowship. WC was supported by an International Senior Research Fellowship from the Wellcome Trust (066646/Z/01/Z). JC is a NHMRC Practitioner Fellow.

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Table Legends

Table 1: Participant Characteristics/demographics at study entry

Table 2: Relationship to HLA-A2 status and DTH induration response (mm) to NY-ESO-1 protein

Table 3: Summary table of subjects demonstrating persisting immunity

Key: nt = not tested
* Persistent disease at entry to follow-up study
**Summary indicates persistence of *any* aspect of NY-ESO-1-specific immunity

Figure Legends

Figure 1. Antibody titer and skin induration in NY-ESO-1 vaccine cohorts. (A) Antibody titers of individual participants grouped according to dose cohort in the initial trial. *y* axis is the reciprocal titer with <2,000 being below the limit of quantitation. **(B)** Skin induration measured two days after intradermal injection of 1 μg of NY-ESO-1 protein without ISCOMATRIX® adjuvant. Each curve represents an individual participant grouped according to dose cohort in the initial trial. Pre-008, before vaccination; Post-008, after three vaccinations at day 86; d2-017, 2 days after protein injection.

Figure 2. Peripheral blood T cell HLA class I- and II-restricted responses to NY-ESO-1 peptides detected from the initial (008) and follow-up (017) trials. The numbers 1-180 at the top and bottom of the figure indicate the amino acid position of NY-ESO-1 protein. Light bars, CD8+ T cell responses; dark bars, CD4+ T cell responses; hatched lines, pre-existing responses detected prior to the initial trial. Numbers to the left indicate the pt number, followed by skin induration response to protein. The letters to the right indicate pt cohort in the initial trial: A= 10μg NY-ESO-1 protein +ISCOMATRIX® dose level; B= 30μg+ ISCOMATRIX® dose level; C= 100μg+ ISCOMATRIX® dose level; D= 100μg without ISCOMATRIX® adjuvant. All patients shown had melanoma except pt 21 who had breast cancer.

Figure 3. Survival and tumour characteristics following NY-ESO-1 vaccination. (A) Kaplan-Meier curve demonstrating relapse-free survival in the subjects with melanoma who were vaccinated with NY-ESO-1/ ISCOMATRIX® versus concurrent placebo. Logrank test; $p < 0.0001$. Median follow-up (all):1430 days, median RFS NY-ESO-1/ISCOMATRIX®: not reached, median RFS placebo: 504 days. Hazard ratio: 0.109 (95% CI: 0.003 – 0.114) **(B)** Antigen and HLA expression prior to vaccination and after relapse demonstrates immuno-editing under the selective pressure of anti-NY-ESO-1 immunity: **i.(left)** Participant 1 (cohort A): IHC for NY-ESO-1 pre-vaccine (017) of subcutaneous nodule. Homogeneous NY-ESO-1 expression and 'brisk' tumor infiltrating lymphocytes noted. **(right)** Participant 1 metastectomy from liver 420 days after vaccination. Persisting NY-ESO-1 and

loss of TILs noted. **ii** Participant 1 S100 (left) and HLA class I (HC-10) (right) indicating loss of class I from liver metastasis. **iii** Participant 12 (cohort C): pre- (left) and post- vaccination (right) loss of NY-ESO-1 expression in relapse sample 122 days post-vaccine. **iv** Participant 9 (cohort C): pre- (left) and post- (right) vaccination loss of NY-ESO-1 expression in relapse sample 750 days post-vaccine. **v.** Participant 13 pre- (left) and post- (right) vaccination loss of NY-ESO-1 expression in relapse sample 919 days post-vaccine.

Characteristics/Demographics		N	%
Number of Subjects Entered	**Total**	**28**	
Sex	Male	15	54
	Female	13	46
Age	Median (range)	55 (34-79)	
No. of days since NY-ESO-1 protein exposure	Median (range)	680 (252-1155)	
Performance Status	100%	24	86
	90%	3	11
	ND	1	4
Previous Therapies	Surgery	28	100
	Radiotherapy	9	32
	Systemic Therapy	9	32
Tumor Stage at study entry	I	2	7
	II	6	21
	III	14	50
	IV	6	21
Tumor Stage (LUD 99-008 vs. LUD 01-017)	*Progressive Stage	6	21
	**Stable Stage	22	79
HLA-A2+		16	57
Treatment Arm (LUD 99-008)	A: vaccine dose 10ug	3	11
	B: vaccine dose 30ug	2	7
	C: vaccine dose 100ug	9	32
	D: NY-ESO-1 protein 100ug without ISCOMATRIX® adjuvant	8	29
	Placebo	6	21
Primary Diagnosis	Breast	3	11
	Melanoma	25	89

*Progressive Stage: Subject had relapse/s between LUD 99-008 and LUD 00-017

**Stable Stage: Subject had no relapse between LUD 99-008 and LUD 00-017

Pt No. in LUD99-008			HLA-A2 POSITIVE SUBJECTS															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total
Pt Cohort			A	A	A	B	B	C	C	C	C	D	D	D	D	P	P	P	16
DTH (mm)		Protein		8					15		5	4		4					5
	Peptides	NY-ESO-1a*		3					4				3					4	3
		NY-ESO-1b**		4	4				3		3		3						5
		NY-ESO-1 DP4***																	0
		gp100																	0

Pt No. in LUD99-008			HLA-A2 NEGATIVE SUBJECTS												
			17	18	19	20	21	22	23	24	25	26	27	28	Total
Pt Cohort			C	C	C	C	C	D	D	D	D	P	P	P	12
DTH (mm)		Protein		12		15	2				6	7		3	6
	Peptides	NY-ESO-1a*													0
		NY-ESO-1b**							3		4			3	3
		NY-ESO-1 DP4***												3	1
		gp100													0

Study Number	Cohort	Persistent or recurrent tumor	Persisting immunity			
			DTH protein	Antibody	T Cell	Summary**
Cohorts vaccinated with NY-ESO-1 in ISCOMATRIX® adjuvant						
1	A	Yes*	x	✓	✓	Yes
2	A	Yes	x	x	x	No
3	A	No	✓	x	✓	Yes
4	B	No	x	x	x	No
6	B	No	x	x	✓	Yes
7	C	No	✓	x	✓	Yes
8	C	No	✓	x	✓	Yes
10	C	No	✓	x	✓	Yes
13	C	Yes	x	x	x	No
16	C	No	x	x	✓	Yes
17	C	No	x	x	✓	Yes
20	C	Yes	x	nt	x	No
21	C	Yes	✓	✓	✓	Yes
24	C	No	x	x	✓	Yes
Subtotal	**14**	**5**	**5**	**2**	**10**	**10**
Cohorts received no adjuvant or no vaccine						
28	D	No	x	x	nt	No
29	D	Yes	x	x	nt	No
30	D	Yes	x	x	nt	No
33	D	Yes	x	x	✓	Yes
37	D	No	x	x	nt	No
39	D	No	x	x	✓	Yes
40	D	No	x	x	x	No
42	D	No	x	x	nt	No
43	P	No	x	x	nt	No
44	P	Yes	x	x	nt	No
45	P	No	x	x	nt	No
49	P	Yes	x	x	nt	No
50	P	Yes	✓	x	nt	Yes
51	P	Yes*	x	x	nt	No
Subtotal	**14**	**7**	**1**	**0**	**2**	**3**

Key:
nt = not tested
* Persistent disease at entry to follow-up study
**Summary indicates persistence of *any* aspect of NY-ESO-1-specific immunity

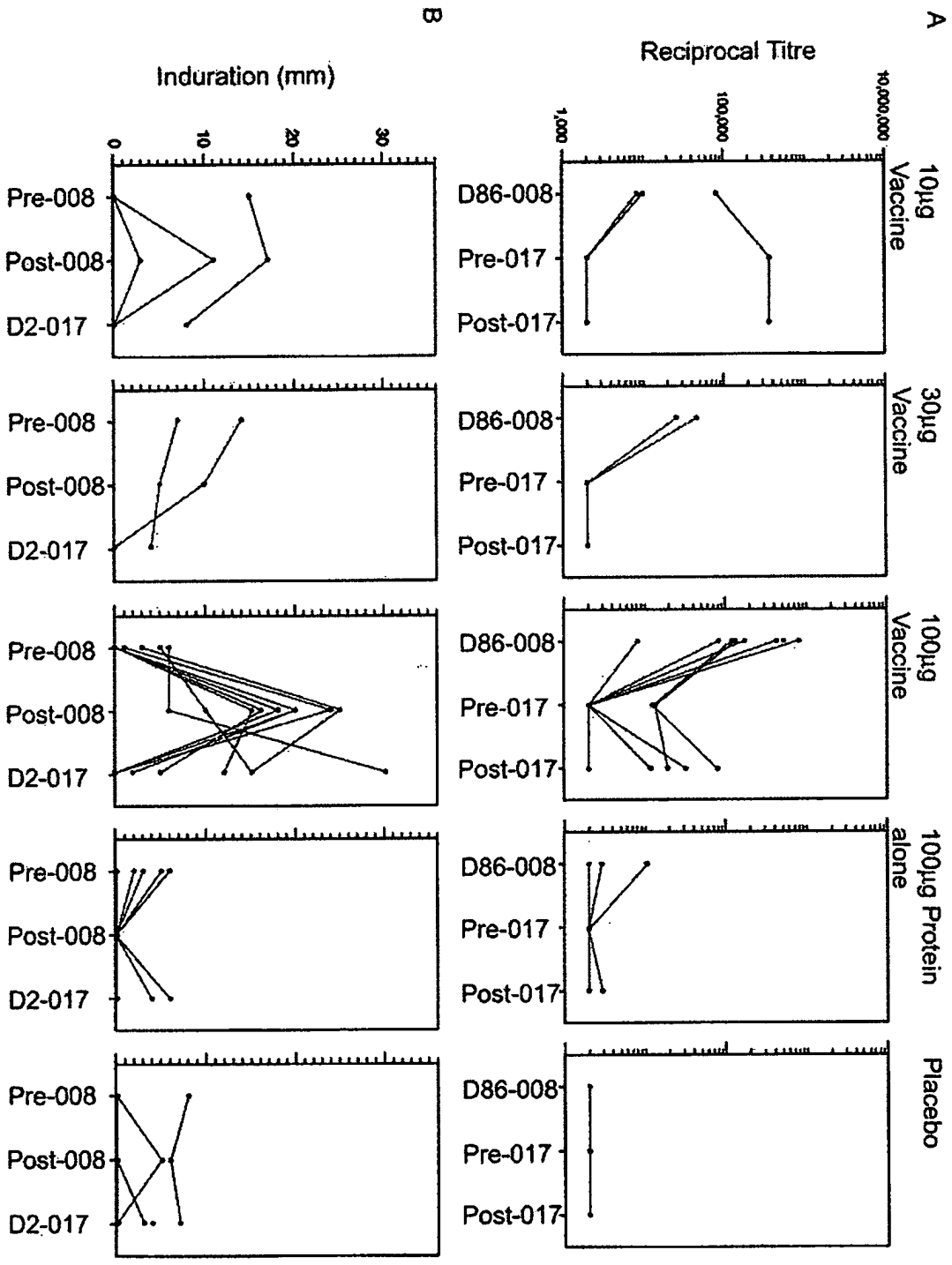
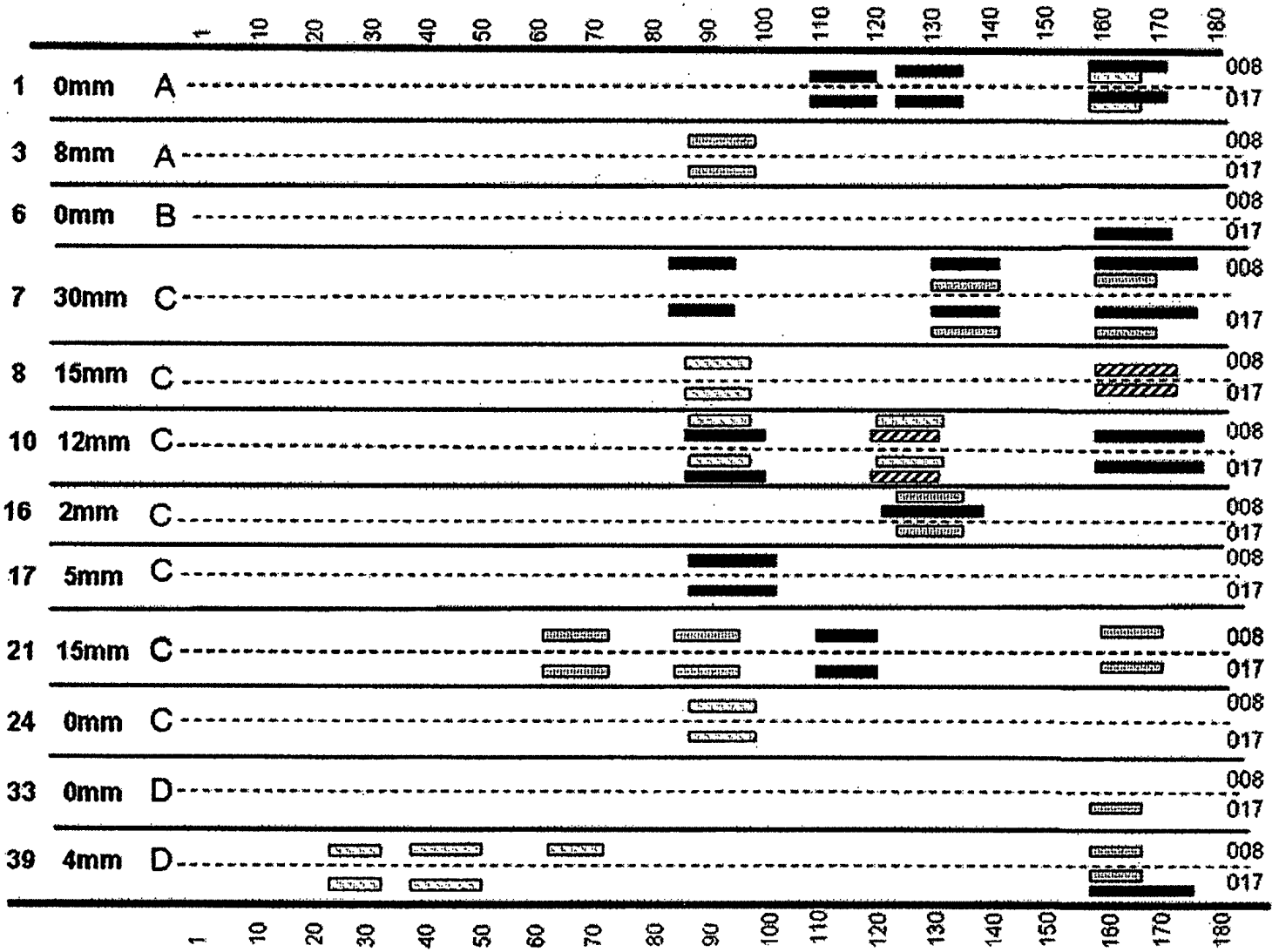


Figure 1 Nicholaou

Nicholaou Figure 2



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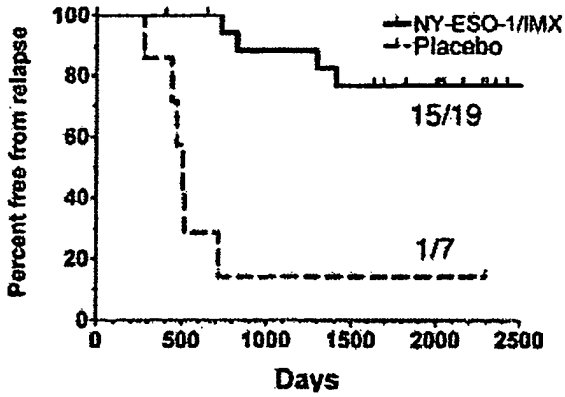

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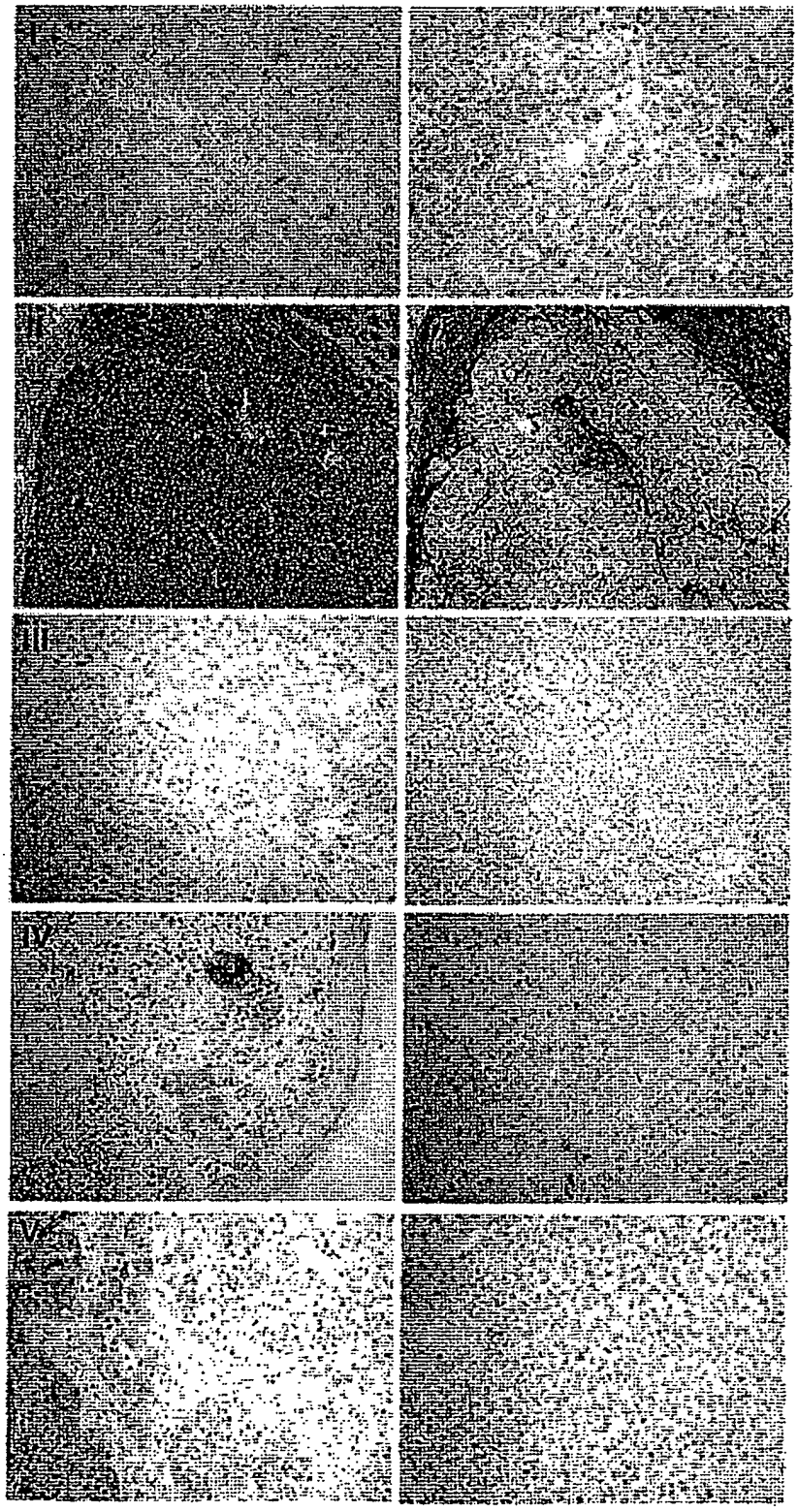

Nicholaou Figure 3